BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

REMARKS

The final Action mailed January 21, 2004, has been carefully studied. The claims in the application remain as claims 1 and 4-25, of which claims 10-23 have been withdrawn from further consideration as being directed to a non-elected invention. The claims examined are claims 1, 4-9, 24 and 25.[1] Applicants again respectfully submit that their claims define novel and unobvious subject matter and should be allowed, whereby applicants respectfully request favorable reconsideration, entry of the amendments presented above, and allowance. Applicants also request withdrawal of the finality of the Office Action of January 21, 2004, as "premature" for the reasons given below at pages 16 and 17.

Claims 1, 4-6, 24 and 25 have been again rejected under §102 as anticipated by Akita, and claims 1 and 9 have been again rejected as obvious under §103 from Akita.[2] These rejections are again respectfully traversed.

---

[1] Paragraph 2 on numbered page 2 of the final Action appears to be in part incorrect, as claim 4 was not previously amended, the total number of claims are greater than as indicated, and it is claims 25 rather than claim 26 which is under examination.

[2] Applicants agree that claim 9 incorporates the subject matter of claim 1. Also, claim 1 encompasses the subject matter of claim 9, but is not limited to the part of claim 9 which appears in dependent form. Applicants continue to maintain that the rejections of claim 1 on both §102 and §103 are inconsistent, not withstanding *In re Best et al,* 195 USPQ 430, 434, note 4 (CCPA 1977) because the rejection in *Best* was **alternatively** under §§102 and 103, not under both sections. Applicants maintain their position of inconsistency as previously pointed out.

First, applicants respectfully repeat by reference what they have already stated regarding Akita (which document, incidentally, comes from the laboratories of the Assignee, it being further noted that the last named of the present applicants is also a co-author of the Akita et al publication). Attached is an annotated clean copy of the Akita et al publication.

The rejection states at page 5, first paragraph, that Akita's Figure 6 does teach that the mAb 25-2G antibody reacts with higher intensity with prohIL-18 and that the intensity is viewed as being equivalent to at least ten times higher as compared with the immunoreactive intensity with mature hIL-18. In view of this, the rejection concludes that rejected claims are anticipated by Akita.

However, it is respectfully submitted that the examiner's understanding of Akita as expressed in the rejection is not correct. Contrary to what is stated in the rejection, and as can be seen from Figure 6A of the attached copy of Akita, immunoreactive intensity of mAb 25-2G antibody with prohIL-18 clearly appears to be lower than that with mature hIL-18, or at worst is no more than the same as that with mature hIL-18. In view of this, it is submitted that mAB 25-2G antibody disclosed in Akita is clearly different from the claimed antibody as defined in claim 1.

From Figure 6B referred to by the examiner, it may be considered that immunoreactive intensity of mAB 25-2G antibody with prohIL-18 is higher than that with mature hIL-18 because the band corresponding to prohIL-18 is denser (i.e. wider) than the band corresponding to mature hIL-18. However, it should be noted that the density of the bands as shown in Figure 6B doe not directly indicate the immunoreactive intensity.

Mature hIL-18 on Figure 6B is formed from prohIL-18 under the action of "partially purified recombinant hICE". Since the formation reaction of mature hIL-18 from prohIL-18 is not complete, the amount of thus formed mature hIL is relatively small. This incompleteness of formation reaction of mature hIL-18 results in less density of the band in Figure 6B. The apparent density of the band corresponding to prohIL-18 looks as though it is higher than that of the band corresponding to mature hIL-18. However, this is just appearance.

Applicants believe and submit that the immunoreactive intensities of mAB 25-2G antibody with mature hIL-18 and prohIL-18 should be evaluated from the density and width of the bands as shown in Figure 6A. The density and width of the band designated as "prohIL-18" in Figure 6Ax (indicated as "X" by hand writing on the attached annotated

copy of Akita), which corresponds to prohIL-18 before "hIL-18-CE" acts on the same, should be compared with those of the band designated as "mature hIL-18" in Figure 6A (indicated as "Y" by handwriting on the attached annotated copy of Akita), which corresponds to "mature hIL-18" fully converted from "prohIL-18" by the action of "hiL-18-CE".

If one supposes from Figure 6B that the immunoreactive intensity of mAb 25-2G antibody with "prohIL-18" is higher than that with "mature hIL-18", then the band corresponding to "prohIL-18" in Figure 6A should be denser and wider than the band corresponding to "mature hIL-18". However, this is not the case. In this regard, it is respectfully submitted that the examiner's assertion is not correct. Akita discloses no similar antibodies other than mAB 25-2G.

The facts are as stated above, and the facts lead to the conclusion that claim 1 (and therefore none of applicant's claims) are anticipated by Akita.

The rejections now appear more clearly to be in part based on assumed inherency, i.e. the PTO appears to assume that because of the data presented in Akita that Akita inherently discloses applicants' isolated antibody to an interleukin-18 precursor. In support of its inherency position, the PTO seeks to shift the burden to applicants to

prove contrary to what is assumed, the PTO relying on *In re Best et al*, 195 USPQ 430 (CCPA 1977). But reliance on *In re Best et al* is inappropriate because the assumed inherency is not inevitable or reasonably certain.

As regards the PTO's reliance on *In re Best et al*, it is inappropriate in part because the evidence in the present case is that the claimed and prior art products are **NOT** identical or substantially identical, nor are they produced by identical or by substantially identical processes, as pointed out above.

In this regard, applicants respectfully revert to the case law such as that cited at pages 12 and 13 of the preceding Reply, which the PTO has overlooked in applying Akita. The law is clear, as appears from *In re Brink*, 164 USPQ 247, 249, that

> Absent a showing [by the PTO] of some reasonable certainty of inherency, the rejection ... under 35 USC 102 must fail.

See also *Ex parte Cyba*, 155 USPQ 756, 757 (1967); and *In re Oelrich*, 212 USPQ 323, 326 (1981); inherency must be "certain" or "inevitable". More recent cases supporting applicants' position in this regard include *Schering Corp v. Geneva Pharmaceuticals, Inc*, 67 USPQ2d 1664 (Fed Cir 2003):

> [A] prior art reference may anticipate without disclosing a feature of the claimed invention if that missing characteristic is **necessarily** present, or

> inherent, in the single anticipating reference. (emphasis added)

Also see the so-called "unpublished" decision in *Holbrooks v. Bacchi*, 69 USPQ2d 1696, 1699 (BPAI 2003) where the Board stated:

> It is not sufficient for inherency that a person following the disclosure of Holbrooks might obtain the result set forth in the claims, it **must invariably happen** (citations omitted; emphasis added)

Again also see *Toro Co v. Deere & Co.*, 69 USPQ2d 1584 (Fed Cir 2004).

The PTO is in no position to validly allege inherency, because inherency is not certain and it is not inevitable, and indeed absolutely does not exist as it is clear from Akita, as pointed out above.

As the rejection based on §103 is in part based on the assumed inherency of the disclosure of Akita, and as that assumed inherency is incorrect, the rejection under §103 must fall for the same reasons.

Applicants again respectfully request withdrawal of the rejection.

The Office Action contains a statement near the bottom of page 4 to the effect that the "industrial usefulness of a product represents the intended use of that product and has no patentable weight." This is not so. The usefulness of

a product, wherein the prior art product does not have that usefulness, is part of the invention "as a whole". In other words, it is an inherent property of that product, an inherent property which the prior art product does not necessarily possesses. Moreover, particularly as regards claim 9 which is directed to a kit, as Akita discloses no motive or incentive for assembling an immunoassay kit, it cannot validly be said that it would have been obvious to do so (as called for in claim 9) from the disclosure of Akita.

This is clearly an obviousness matter, and Akita does not disclose anything which would have caused the person of ordinary skill in the art to assemble an immunoassay kit embodying any material of Akita. Stated in yet another way, the teaching of a kit comes only from **applicants'** disclosure, not from any other prior art applied.

The rejection of claim 9 should also be withdrawn for these additional reasons, and such is respectfully requested.

Claims 1, 4-9, 24 and 25 have been rejected under the second paragraph of §112. The rejection is respectfully traversed.

Applicants believe that the claims as previously drafted, considered in light of applicants' specification

(fully consistent with the law), would not have been confusing to those skilled in the art, and therefore the claims in their previous form are fully in accordance with §112. At **worst**, claim 1 in its previous form might be considered objectionable, but **only** as to form, requiring no substantial amendment relating to patentability.

Nevertheless, in deference to the examiner's views and to avoid needless argument, applicants propose to amend claim 1 as submitted above. Such an amendment is considered to be of a formal cosmetic nature only, i.e. made to place the claims in improved form consistent with the examiner's understanding of what is necessary or desirable under U.S. practice. Applicants submit that the amendments proposed are not "narrowing" amendments because the scope of the claims is not reduced by such amendments.

To elaborate upon what is stated above, the amendment proposed above for claim 1 merely replaces the word "specific" with the definition of what "specific" means in the context of the present invention, support being found in applicant's specification at page 7, lines 16-20.

Applicants respectfully request entry of the amendments presented above and withdrawal of the rejection.

The claims have also been rejected under the first paragraph of §112 for substantially the same reasons as given for the rejection of claim 1 under the second paragraph of §112. This rejection is respectfully traversed.

The rejection indicates that the phrase in claim 1, "a higher immunoreactivity to said interleukin 18 precursor than to mature interleukin 18 by at least 10 times" is not in conformity with the description at page 35, lines 6-8, "Each antibody exhibited an immunoreactivity against human IL-18 only at about 10 to about 2% or less of that against human IL-18 precursor". However, the applicant does not agree with the rejection for the following reasons.

It should be noted that the description pointed out in the rejection refers "about 10 to about 2%". "10%" corresponds to 1/10 of 100% and "2%" corresponds to 1/50 of 100%. The description therefore states that each antibody exhibited immunoreactivity against human IL-18 precursor about 10 to 50 times of that against human IL-18.

Furthermore, the specification at page 7, line 6 from the bottom to page 8, line 1, states as follows;

> "For example, when the levels of the immunoreactivity of the present antibody are compared between the cases against mature L-18 and IL-18 precursor, the immunoreactivity against mature IL-18 is apparently lower than that against the precursor; at an intensity of, usually, at most 10%,

preferably, at most 2%, and more preferably, at most 1% of that against the precursor."

This statement corresponds to what we submit.

It is therefore believed that the rejection under 35 U.S.C. §112, first paragraph, has no reasonable grounds.

Again, the proposed amendment to claim 1 is of a formal nature only, as pointed out above (please again see page 7, lines 16-20 of applicants' specification) and does not change the scope of the claims.

As the term "specific" is fully supported in applicant's specification, and means exactly the same thing as what is meant by the addition to claim 1 as proposed above, no new matter and no new issue are involved, whereby applicants again respectfully request entry of the amendments presented above, as well as withdrawal of the rejection under the first paragraph of §112.

Claims 1, 4-8 and 24 have been rejected under §103 as obvious from Yong in view of Campbell (see paragraph 23 of the final rejection). This rejection is respectfully traversed.

This is a new rejection which clearly was **not** necessitated by any amendments presented in the last Reply by the applicants. Accordingly, applicants request withdrawal of the finality of the Office Action mailed January 21, 2004.

As to the merits of this new rejection, the rejection asserts that it would have been obvious to one of ordinary skill in the art to generate and isolate a monoclonal or polyclonal antibody to Yong's purified interleukin 18 precursor expressed in E. coli using art-known antibody production techniques such as shown in Campbell.

The applicants, however, respectfully disagree strongly. It is apparent to one of ordinary skill in the art that it requires undue experimentation to obtain the desired antibody. In fact, Akita et al failed to obtain the claimed antibody.

Furthermore, it is submitted that Yong does not provide any motivation to obtain an antibody of "a purified recombinant human IL-18 precursor expressed in E. coli". Neither does Campbell. Therefore, even if the proposed combination were obvious, not conceded by applicants, such combination would not reach or even lead to the claimed subject matter.

Withdrawal of this new rejection is in order and is respectfully requested.

Claims 9 and 25 have also been rejected as obvious under §103 from Yong in view of Campbell. This rejection is respectfully traversed for the same reasons as indicated above

relative to the rejection of claim 1 as obvious from Yong in view of Campbell.

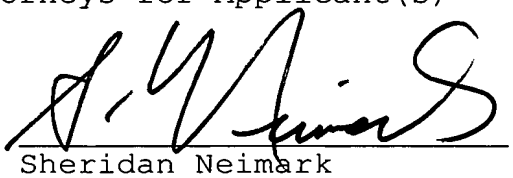
Moreover, as the dependent portions of claim 9 and 25 add additional features, and as these additional features are not expressly taught or made obvious by the applied prior art, claims 9 and 25 define additional non-obvious subject matter over the prior art.

Applicants respectfully request withdrawal of this rejection.

Applicants again request favorable reconsideration, entry of the amendments presented above and formal allowance.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant(s)

By 
Sheridan Neimark
Registration No. 20,520

SN:jaa/ma
Telephone No.: (202) 628-5197
Facsimile No.: (202) 737-3528
G:\BN\S\SUMA\Kayano 1\PTO\AMD 12JL04.doc

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 42, Issue of October 17, pp. 26595–26603, 1997
Printed in U.S.A.

Involvement of Caspase-1 and Caspase-3 in the Production and Processing of Mature Human Interleukin 18 in Monocytic THP.1 Cells*

(Received for publication, July 15, 1997, and in revised form, July 31, 1997)

Kenji Akita‡, Takashi Ohtsuki, Yoshiyuki Nukada, Tadao Tanimoto, Motoshi Namba, Takanori Okura, Rohko Takakura-Yamamoto, Kakuji Torigoe, Yong Gu§, Michael S.-S. Su§, Mitsukiyo Fujii, Michiyo Satoh-Itoh, Kouzo Yamamoto, Keizo Kohno, Masao Ikeda, and Masashi Kurimoto

From the Fujisaki Institute, Hayashibara Biochemical Laboratories, Inc., 675-1 Fujisaki, Okayama 702, Japan, and §Vertex Pharmaceuticals Incorporated, Cambridge, Massachusetts 02139

Recently, human interleukin 18 (hIL-18) cDNA was cloned, and the recombinant protein with a tentatively assigned NH_2-terminal amino acid sequence was generated. However, natural hIL-18 has not yet been isolated, and its cellular processing is therefore still unclear. To clarify this, we purified natural hIL-18 from the cytosolic extract of monocytic THP.1 cells. Natural hIL-18 exhibited a molecular mass of 18.2 kDa, and the NH_2-terminal amino acid was Tyr^{37}. Biological activities of the purified protein were identical to those of recombinant hIL-18 with respect to the enhancement of natural killer cell cytotoxicity and interferon-γ production by human peripheral blood mononuclear cells. We also found two precursor hIL-18 (prohIL-18)-processing activities in the cytosol of THP.1 cells. These activities were blocked separately by the caspase inhibitors Ac-YVAD-CHO and Ac-DEVD-CHO. Further analyses of the partially purified enzymes revealed that one is caspase-1, which cleaves prohIL-18 at the Asp^{36}-Tyr^{37} site to generate the mature hIL-18, and the other is caspase-3, which cleaves both precursor and mature hIL-18 at Asp^{71}-Ser^{72} and Asp^{76}-Asn^{77} to generate biologically inactive products. These results suggest that the production and processing of natural hIL-18 are regulated by two processing enzymes, caspase-1 and caspase-3, in THP.1 cells.

Interleukin (IL)[1]-18 (originally called IGIF, interferon-γ-inducing factor) is a novel cytokine with multiple biological functions. In 1995 we purified murine IL-18 from the liver extracts of mice sensitized with *Propionibacterium acnes* followed by elicitation with lipopolysaccaride (1). The cDNA of murine IL-18 was cloned from cDNA libraries prepared from the livers of mice with endotoxin shock (2). Using this as a probe, human IL-18 cDNA was also cloned from a human normal liver cDNA library (3). The recombinant human IL-18 with a tentatively assigned NH_2-terminal amino acid based on its homology with the natural murine IL-18 sequence was expressed in *Escherichia coli*, and its biological activities were examined (3).

IL-18 has an interleukin 1 (IL-1) signature-like sequence (3) as reported and is similar to the IL-1 family and fibroblast growth factor in terms of their trefoil structures (4, 5). Despite their similarities, IL-18 and IL-1β exhibit different biological activities (2, 3, 6), transmitted through their specific receptors.[2] Genetic information suggested that IL-18 is synthesized as an inactive precursor form (prohIL-18) and that this prohIL-18 has no known signal peptide sequence. Therefore, proteolytic cleavage is required for its maturation like IL-1β (2, 3, 7, 8). Gu *et al.* (7) reported that IL-1β-converting enzyme (ICE)/caspase-1 cleaved murine proIL-18 at the authentic processing site, Asp^{35}-Asn^{36}, to generate biologically active mature murine IL-18. However, natural hIL-18 had not yet been isolated, and its maturation site remained unclear.

In this report, we screened for hIL-18 mRNA-expressing cell lines and purified natural hIL-18 from the cell-free extract of positively expressing cells. Furthermore, we identified two hIL-18-processing enzymes in the same cellular extract. One enzyme is ICE/caspase-1, which acts on prohIL-18 to generate the mature active form of hIL-18, and the other is CPP32/caspase-3, which acts on both the prohIL-18 and the mature hIL-18 to generate biologically inactive degraded products. This is the first report on the identification of natural hIL-18 and its processing enzymes existing in the same cells.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies

Tetrapeptidyl ICE-like protease inhibitor Ac-YVAD-CHO and CPP32-like protease inhibitor Ac-DEVD-CHO, and ICE- and CPP32-like protease fluorogenic substrates Ac-YVAD-MCA and Ac-DEVD-MCA, respectively, were purchased from the Peptide Institute (Osaka, Japan). Neutralizing and non-neutralizing anti-hIL-18 murine monoclonal antibodies (mAbs) 125-2H (IgG) and 25-2G (IgG) were raised against the recombinant protein in our laboratory. Human IL-1β precursor and anti-precursor human IL-1β rabbit polyclonal antibody (pAb) were obtained from Cistron (Pine Brook, NJ). Anti-human ICE-p20 subunit, anti-human CPP32, anti-human poly(ADP-ribose)polymerase goat pAbs, and anti-human ICE-p10 subunit rabbit pAb were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Recombinant purified human $(His)_6$-tagged CPP32, $(His)_6$-tagged CMH-1,

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel: 81-86-276-3141; Fax: 81-86-276-6885; E-mail: Fujih@po.harenet.or.jp.

[1] The abbreviations used are: IL, interleukin; pro, precursor; h, human; ICE, interleukin-1β-converting enzyme; mAb, monoclonal antibody; pAb, polyclonal antibody; PCR, polymerase chain reaction; PB, phosphate buffer; IFN, interferon; NK cell, natural killer cell; ICA, human IL-18-converting activity; IDA, human IL-18-degrading activity; hIL-18-CE, human IL-18-converting enzyme; hIL-18-DE, human IL-18-degrading enzyme; CHAPS, 3-[(3-cholamidpropyl)dimethylammonio]-1-propanesulfonic acid; DTT, dithiothreitol; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; PBMC, peripheral blood mononuclear cells.

[2] Torigoe, K., Ushio, S., Okura, T., Kobayashi, S., Taniai, M., Kunikata, T., Murakami, T., Sanou, O., Kojima, H., Fujii, M., Ohta, T., Ikeda, M., Ikegami, H., and Kurimoto, M. (1997) *J. Biol. Chem.*, in press.

and anti-CMH-1 rabbit antiserum (antibody 25) were prepared at Vertex Pharmaceuticals Inc. (Cambridge, MA).

Cell Culture

42 hematopoietic cell lines were cultured for the screening of hIL-18 mRNA in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Whittaker, Walkersville, MD), 100 units/ml penicillin, and 50 μg/ml streptomycin. Cells were cultured under standard conditions in a humidified 5% CO_2 and air mixture at 37 °C. To obtain large amounts of THP.1 cells, an *in vivo* cell propagation method using immunosuppressed hamsters was applied as described previously (9, 10). Briefly, 5×10^6 THP.1 cells were transplanted subcutaneously into newborn hamsters and injected intraperitoneally with 0.1 ml of a rabbit anti-hamster thymocyte serum (J R Scientific, Inc., Woodland, CA). Every 3 or 4 days after the transplantation, these hamsters were given 0.1 ml of the serum intraperitoneally. After 3 weeks, the solid tumor masses of THP.1 cells were harvested, rinsed thoroughly with RPMI 1640 medium, and small tumor masses were dispersed by scissors and then passed through stainless steel meshes to obtain a single cell suspension.

hIL-18 mRNA Screening Using Reverse Transcription-PCR

Total RNAs were isolated from various cell lines by ULTRASPEC-3 (Biotecx, Houston, TX) in accordance with the supplier's instructions. The RNAs were reverse transcribed and amplified by PCR. The thermocycle conditicns were 30 or 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, for denaturation, annealing, and extension, respectively. The sequences of the primer pair for hIL-18 were 5′-GCTTGAATCTAAATTATTATCAGTC-3′ and 5′-CAAATTGCATCT-TATTATCATG-3′, which produced a 335-base pair product. The PCR products were run on 3% agarose gels and visualized with ethidium bromide staining.

Purification of Natural hIL-18

All purification steps were performed at 4 °C except for DEAE-5PW chromatography, which was carried out at room temperature.

Step 1: Preparation of a Cell-free Extract of THP.1—A THP.1 cell-free extract was prepared by hypotonic lysis (11). Briefly, the cell pellets from 5×10^{11} THP.1 cells were washed once with 10 volumes of hypotonic buffer (20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA·2Na) and were collected by centrifugation at $1,000 \times g$ for 10 min. The resulting cell pellets were resuspended in 3 volumes of hypotonic buffer and frozen at −20 °C until use. Cells were disrupted by three cycles of freeze-thawing, and cell debris was removed by centrifugation at $10,000 \times g$. The cell-free extract obtained was then dialyzed against 10 mM phosphate buffer (PB) at pH 6.6.

Step 2: DEAE-Sepharose Fast Flow Chromatography—The cell-free extract (7.2 liters) from step 1 was loaded onto a 3.6-liter DEAE-Sepharose Fast Flow column (Pharmacia Biotech Inc.) equilibrated with 10 mM PB at pH 6.6. After washing with the same buffer, the extract was eluted with 10 mM PB containing 0.2 M NaCl and then 0.5 M NaCl. The fractions containing hIL-18 activity eluted with 0.2 M NaCl (8.0 liters) were pooled and concentrated to 1.0 liter with a hollow fiber membrane module AIP-1010 model (Asahi Chemical Industry, Tokyo, Japan) and dialyzed overnight against 20 mM PB at pH 6.8.

Step 3: DEAE-5PW Chromatography—The dialyzed product from step 2 was passed through a 0.22-μm Millipak filter (Millipore, Bedford, MA), loaded onto a 550-ml DEAE-5PW column (Tosoh, Tokyo) equilibrated with 20 mM PB at pH 6.8, and washed with the same buffer. Elution was carried out with 5 bed volumes of a linear salt gradient (0–0.5 M NaCl), and 25-ml elute fractions were collected. hIL-18 activity was eluted at 90–200 mM NaCl in 20 mM PB, and fractions 44–54 containing the activity were pooled and concentrated to 50 ml with hollow fiber membranes, Mini-module model NM-3 (Asahi Chemical Industry).

Step 4: Affinity Chromatography—A hIL-18 affinity column was prepared using CNBr-activated Sepharose 4B (Pharmacia) and anti-hIL-18 murine mAb (125-2H) in our laboratory. The pooled and concentrated preparation from step 3 was applied onto a 1-ml hIL-18 affinity column equilibrated with phosphate-buffered saline at pH 7.2. After washing with the same buffer extensively, the sample was eluted with 100 mM glycine-HCl buffer containing 0.1 M NaCl at pH 2.5. Eluted fractions were neutralized immediately with 1.0 M Tris.

Step 5: Reverse Phase C_4 Chromatography—The hIL-18-containing fractions from step 4 were loaded onto a 4.2-ml reverse phase ASAHIPAK C_4P-50 4E column (Showa Denko, Tokyo), equilibrated with 0.1% trifluoroacetic acid, and the column was washed with the same buffer. Next, the sample was eluted with 24 bed volumes of an increasing acetonitrile linear gradient (0–90% acetonitrile). The main peak fraction eluted at about 40% acetonitrile and was used for peptide mapping of natural hIL-18.

Biological Analysis of Natural hIL-18

IFN-γ production assay and NK cell cytotoxicity assay were performed as described previously (3).

Separation of hIL-18-converting Activity (ICA) and hIL-18-degrading Activity (IDA)

As in the previous procedures, all purification steps were performed at 4 °C except for DEAE-5PW chromatography, which was carried out at room temperature.

Step 1: Preparation of a Cell-free Extract of THP.1—THP.1 cells (2.0×10^{11} cells) were obtained as described above. Cell-free extract was prepared by the method of Miller *et al.* (12).

Step 2: Ammonium Sulfate Fractionation—Granular ammonium sulfate was added to the extract to reach 40% saturation. The solution was stirred for 20 min, and the resulting precipitate was removed by centrifugation. The supernatant was then brought to 80% saturation with ammonium sulfate, and the precipitate collected by centrifugation was resuspended in TGCD buffer (20 mM Tris, pH 7.8, 5% glycerol, 0.1% CHAPS, 2 mM dithiothreitol (DTT)), and dialyzed against the same fresh buffer for 16 h.

Step 3: DEAE-5PW Chromatography—The dialyzed fraction from step 2 was passed through a 0.22-μm poly(vinylidene fluoride) membrane filter (Millipore). Half of the preparation was loaded onto a 550-ml DEAE-5PW column equilibrated with TGCD buffer and washed with the same buffer. The extract was eluted in two batches of 45-ml fractions with 4.5 bed volumes of a linear salt gradient (0–0.25 M NaCl). Fractions containing ICA (fractions 17–21 eluting at about 100 mM NaCl) and IDA (fractions 33–48 eluting at 150–210 mM NaCl) were pooled separately and dialyzed against HGCD buffer (20 mM Hepes, pH 7.2, 5% glycerol, 0.1% CHAPS, 2 mM DTT). These two preparations were subjected to further purification.

Partial Purification of ICA

Step 1: S-Sepharose Chromatography—Half of the dialyzed fraction containing ICA was loaded onto a 20-ml S-Sepharose column (Pharmacia) equilibrated with HGCD buffer and washed with the same buffer. Then the sample was eluted in two batches of 5-ml fractions with 6 bed volumes of a linear salt gradient (0–0.12 M KCl). Fractions 23–26 containing ICA eluted at 50–80 mM KCl in HGCD buffer and were pooled and dialyzed against HGCD buffer.

Step 2: Concentration using Mono S Column—This microvolume concentration step was carried out with a SMART system (Pharmacia) in accordance with the supplier's instructions. One-third of the dialyzed fractions containing ICA was loaded onto a 0.1-ml Mono S column (Pharmacia) equilibrated with HGCD buffer and washed with the same buffer. Then the sample was eluted in three batches of 25-μl fractions with an HGCD buffer containing 0.5 M KCl. Fractions 3–5 containing ICA were pooled and used in the next process.

Step 3: Superdex 200 Gel Filtration—One-third of the pooled fractions from step 2 was loaded onto a 2.4-ml Superdex 200 column (Pharmacia) equilibrated with HGCD buffer. The sample was eluted in three batches of 80-μl fractions with the same buffer. ICA was eluted in fractions 11–14. Biologically active fractions were pooled and subjected to NH_2-terminal analysis of the polypeptides.

Partial Purification of IDA

Step 1: S-Sepharose Chromatography—One-third of the dialyzed fraction containing IDA was loaded onto a 20-ml S-Sepharose column equilibrated with HGCD buffer at pH 7.0 and washed with the same buffer. The sample was then eluted in three batches of 10-ml fractions with HGCD buffer containing 0.5 M KCl. Fractions 3–5 containing IDA were pooled and dialyzed against 25 mM Bis-Tris buffer at pH 7.1.

Step 2: Mono P Chromatofocusing—Half of the dialyzed pool from step 1 was loaded onto a 20-ml Mono P column (Pharmacia) equilibrated with 25 mM Bis-Tris buffer at pH 7.1 and washed with the same buffer. The sample was eluted in two batches of 8-ml fractions with 10% polybuffer (Pharmacia) and iminodiacetic acid at pH 5.0, which generates a decreasing linear pH gradient (pH 7.1–5.0). Fractions containing IDA (pH 5.8–6.0) were pooled and dialyzed against HGCD buffer.

Step 3: Superdex 200 Gel Filtration—The dialyzed pooled fractions from step 2 were concentrated and loaded onto a 120-ml Superdex 200 column (Pharmacia) equilibrated with HGD buffer (HGCD buffer with-

out CHAPS) at pH 7.4. IDA was eluted in fractions 29–35. Fractions containing IDA were pooled and used in next process.

Step 4: Hydroxyapatite Chromatography—The pooled fractions from step 3 were loaded in two batches onto a 4.4-ml hydroxyapatite column (Tonen, Tokyo) equilibrated with HGD buffer at pH 7.4. The sample was then eluted in 0.5-ml fractions with 5 bed volumes of a linear phosphate gradient (0–0.5 M). Fractions containing IDA eluted at 500 mM phosphate in HGD buffer in fractions 54 and 55, which were pooled and subjected to NH_2-terminal analysis of the polypeptides.

NH_2-terminal Analysis and Peptide Mapping

Amino acid sequences of the NH_2-terminal portions of purified natural hIL-18 and processed forms of hIL-18 (p18, p16, and p15) and of partially purified hIL-18-processing enzymes were determined with an automated protein sequencer model 473A (Applied Biosystems, Foster City, CA). Briefly, proteins of interest were subjected to electrophoresis in 15% SDS-PAGE gels and transferred onto ProBlott™ membranes (Applied Biosystems). The proteins were visualized by Coomassie Brilliant Blue staining and excised for direct NH_2-terminal sequencing. For peptide mapping, purified natural hIL-18 was digested with clostripain (Sigma), and the resulting peptide fragments were separated using a 2.5-ml ODS-120T column (Tosoh) with 20 bed volumes of increasing acetonitrile linear gradient (0–70% acetonitrile) in 0.1% trifluoroacetic acid. Eluted fragments detected at a wavelength of 214 nm were separately collected and analyzed.

Preparation of Precursor hIL-18

A 0.8-kilobase cDNA encoding precursor hIL-18 (3) was ligated into the expression vector BCMGSNeO (13). The hIL-18 expression vector was transfected into CHO-K1 cells by electroporation, and transfectants were harvested after 3 days. The expressed protein was analyzed by immunoblotting using a hIL-18-specific mAb (25-2G).

Preparation of Recombinant Human ICE (rhICE)

Human ICE cDNA was cloned from THP.1 cells using the PCR method. PCR primers were designed based on the published hICE nucleotide sequence (14). COS-1 cells were transfected by electroporation with an expression construct containing hICE cDNA into pCDM8 expression vector (Invitrogen, San Diego) and harvested after 3 days. Proteins from the cells were extracted with hypotonic buffer using a Dounce homogenizer. The supernatant of this homogenate was incubated at 37 °C for 1.5 h to activate ICE precursor and was partially purified by ammonium sulfate fractionation followed by DEAE-5PW and Superdex 75 column chromatographies.

hIL-18 Assay

The quantities of hIL-18 were assessed by a two-site sandwich ELISA using mAbs prepared in our laboratory (15). The bioactivity of hIL-18 was assessed by its IFN-γ-inducing activity on the myelomonocytic cell line KG-1.[3] Briefly, 3×10^5 KG-1 cells suspended in RPMI 1640 medium containing 10% fetal bovine serum were seeded in a 96-well microplate. Samples were added to the wells and incubated for 24 h, and IFN-γ activity induced in the culture supernatants was assessed by ELISA.

Measurements of ICE-like and CPP32-like Activities

ICE-like and CPP32-like activities were measured using the fluorogenic substrates Ac-YVAD-MCA and Ac-DEVD-MCA, respectively (12, 14, 18). Briefly, 1 mM substrate and samples (10 μl) were incubated at 37 °C in the presence of 20 mM Hepes, 10% glycerol, and 2 mM DTT in 96-well microplates (100 μl) at pH 7.4 for 3 h (ICE-like activity assay) or at pH 6.5 for 0.5–1 h (CPP32-like activity assay). The levels of generated 4-amino-2-methylcoumarin were assessed with a fluorescence plate reader (FluoroScan II, Labsystems Japan, Tokyo) at a wavelength of 355 nm for excitation and 460 nm for emission. The activities of these proteases were calculated and normalized as pmol of 4-amino-2-methylcoumarin formed/μl/min.

RESULTS

hIL-18 mRNA Is Predominantly Detected in Myelomonocytic Cell Lines—To identify hIL-18-producing cell lines, we first examined the level of constitutive expression of hIL-18 mRNA by reverse transcription-PCR analysis using hIL-18-specific primers. 42 hematopoietic cell lines of various lineages were screened. hIL-18 mRNA was highly expressed in myelomonocytic cell lines such as THP.1, KG-1, HBL-38, and HL-60, and some non-lymphocytic and non-myelomonocytic cell lines such as MEG-01 and TS9;22. In some B cell and non-T, non-B cell lines such as BJAB, MOLP-2, and NALM-20, weak expression of hIL-18 mRNA was observed. Interestingly, no expression was observed in T cell lines (Table I).

TABLE I

Constitutive expression of hIL-18 mRNA in hematopoietic cell lines

The levels of constitutive expression of hIL-18 mRNA were examined by reverse transcription-PCR analysis using IL-18-specific primers. 42 hematopoietic cell lines of various lineages were studied. The strength of expression was judged from the density of the band detected: very strongly expressed, +++; strongly expressed; ++; weakly expressed; +; no expression, −.

Cell lines	PCR cycles	
	30	35
T cell		
DND-39	−	−
HUT-78	−	−
C5/MJ	−	−
SAALT-3	−	−
DND-41	−	−
JM	−	−
JURKAT	−	−
PEER	−	−
ED-S	−	−
B cell		
NALM-6	−	−
BALM-9	−	−
BALM-10	−	−
MKB-1	−	−
Hair-M	−	−
HPB-ALL	−	−
DAUDI	−	−
RAJI	−	−
BAL-KHs	−	−
BALL-1	−	−
B372	−	−
B373	−	−
B374	−	−
Ramos	−	+
MLB-1084	−	+
MOLP-2	−	++
BJAB	−	++
Non-T, non-B cell		
HDLM-2	−	−
NALM-19	−	−
NALM-20	−	++
NALM-24	−	++
Myelomonocyte cell		
KCL-22	−	−
U-937	+	++
ML-1	+	++
KU-812	+	++
THP.1	+	+++
HL-60	+	+++
KG-1	++	+++
HBL-38	++	+++
Non-L, non-M cell		
L428	−	−
HEL	+	++
MEG-01	+	+++
TS9; 22	+	+++

Purification of Natural hIL-18—Initially, we chose four myelomonocytic cell lines (THP.1, HL-60, U-937, and HBL-38) and two non-L, non-M cell lines (HEL and MEG-01) as targets for the detection of natural hIL-18 at the protein level. As a preliminary study, culture supernatants or extracts of these cell lines were analyzed using hIL-18-specific ELISA and immunoblotting. Small amounts of hIL-18 were detected in the cell extracts of THP.1 and U937 cells, but little or no hIL-18 was detected in the culture supernatants (data not shown). Furthermore, hIL-18 was found mostly in the cytosolic fraction of

[3] K. Konishi, F. Tanabe, M. Taniguchi, H. Yamauchi, T. Tanimoto, M. Ikeda, K. Orita, and M. Kurimoto, submitted for publication.

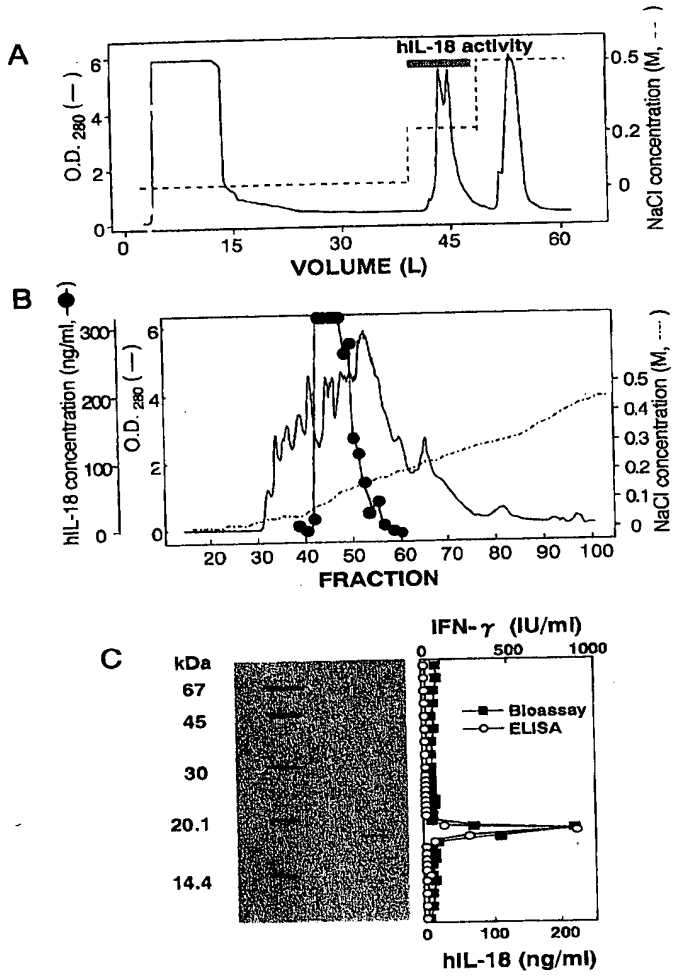


FIG. 1. **Purification of natural hIL-18.** *Panel A*, DEAE-Sepharose Fast Flow chromatography. The cell-free extract (7.2 liters) from THP.1 cells was loaded onto a 3.6-liter DEAE-Sepharose Fast Flow column as described under "Experimental Procedures." hIL-18 activity was assessed by ELISA. The *shaded bar* indicates active fractions that were pooled for the next purification step. *Dashed line*, NaCl concentration; *solid line*, A_{280} absorption. *Panel B*, DEAE-5PW chromatography. The pooled fractions from DEAE-Sepharose chromatography were loaded onto a 550-ml DEAE-5PW column as described under "Experimental Procedures." *Dashed line*, NaCl concentration; *solid line*, A_{280} absorption; *closed circles*, hIL-18 concentration. *Panel C*, SDS-PAGE analysis of affinity-purified natural hIL-18. Coomassie Brilliant Blue staining (*left*) and hIL-18 activity extracted from sliced polyacrylamide gel fragments (*right*) are shown. Sliced gel fragments (2- or 5-mm thickness) were soaked in 0.5 or 1.25 ml of RPMI 1640 medium containing 10% fetal bovine serum for 12 h at 4 °C, and then supernatants were subjected to ELISA (*open circles*) and IFN-γ-inducing assay on KG-1 cells (*closed squares*).

the cells. Next we employed some differentiation-inducing agents (lipopolysaccaride, retinoic acid, A23187, and phorbol myristate acetate) to investigate whether these reagents can augment the production or secretion of hIL-18. However, no significant elevation in the levels of intra- or extracellular hIL-18 was observed (data not shown). Based upon the above mentioned preliminary results, we selected the cytosolic extract of THP.1 cells as a starting material for the isolation of natural hIL-18. For this purpose, large numbers of THP.1 cells were obtained with an *in vivo* cell propagation method (9, 10), and a cytosolic extract was prepared by hypotonic lysis. The cell

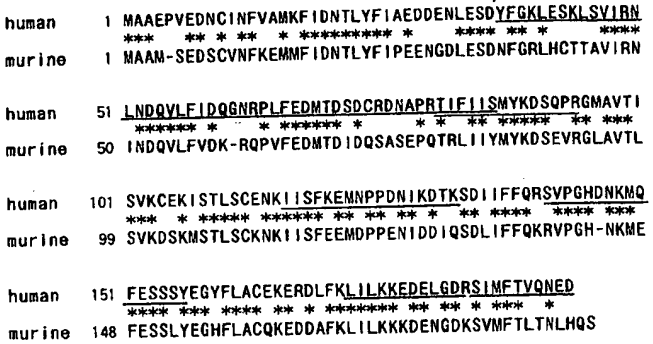


FIG. 2. **Amino acid sequences of hIL-18 and alignment with the murine homolog.** Alignment of the protein sequences of human and murine IL-18 is shown. *Asterisks* (*) denote residues that are identical in the two sequences. The *vertical arrow* indicates the NH_2 terminus of the maturation site of natural hIL-18 at which proteolytic processing occurs. Partial sequences of natural hIL-18 confirmed by Edman sequencing are *underlined*. The starting residues of each sequence are numbered to the *left* of the sequence.

extract was subjected to DEAE anion-exchange chromatography twice (Fig. 1, *A* and *B*) for the purification of natural hIL-18. Subsequently, affinity purification on anti-hIL-18 mAb-conjugated Sepharose was employed. The purified protein had a molecular mass of 18.2 kDa on SDS-PAGE under reducing conditions and exhibited IFN-γ-inducing activity on KG-1 cells (Fig. 1*C*). The overall yield of natural hIL-18 after affinity purification ranged from 3 to 30 $\mu g/10^{11}$ cells. Direct amino acid sequencing of the affinity-purified polypeptide showed that the NH_2-terminal amino acid is Tyr^{37}. Furthermore, the amino acid sequences of the peptides prepared from the purified polypeptide coincided with those deduced from the cDNA (Fig. 2; *underlined*). These results indicate that the purified polypeptide derived from THP.1 is a mature form of hIL-18 proteolytically processed behind the Asp^{36} residue in a manner similar to that of the murine homolog (Fig. 2; *vertical arrow*) (1, 2, 6).

Biological Analysis of Natural hIL-18—To confirm the biological activities of the protein purified from THP.1, IFN-γ-inducing ability and enhancement of NK cell cytotoxicity of the natural hIL-18 were examined on human PBMC. In the presence of anti-CD3 mAb, a concentration as low as 5.5 pM of either natural or recombinant hIL-18 significantly augmented IFN-γ production by PBMC (Fig. 3*A*). Pretreatment of PBMC with natural hIL-18 enhanced NK cytotoxicity against a target cell line, K562, in a manner similar to that of the recombinant hIL-18 (3). The enhancement of NK cell cytotoxicity was observed at concentrations as low as 0.55 pM (Fig. 3*B*). These results suggest that the purified natural hIL-18 is equivalent to the recombinant protein in its biological functions.

THP.1 Cell Lysate Contains Two Types of ProhIL-18-processing Activities—Besides the natural hIL-18, the THP.1 cell-free extract also contained the precursor protein as determined by immunoblotting (data not shown). This suggests that proteolytic maturation occurs in THP.1 cells or in the cell-free extract during the purification process. The amino acid sequence of the hIL-18 maturation site (Asp^{36}-Tyr^{37}) seems to be specific to caspases (16) or granzyme B (17), suggesting the presence of an IL-18-converting enzyme (hIL-18-CE) in the cytosolic extract of THP.1 cells. To confirm this, the recombinant precursor protein of hIL-18 (prohIL-18; p24) was incubated for various periods with the THP.1 cell extract. Immunoblotting using hIL-18-specific antibody (25-2G) revealed that prohIL-18 was proteolytically cleaved by the THP.1 extracts to generate three cleavage products with molecular masses of 18 kDa (p18), 16 kDa

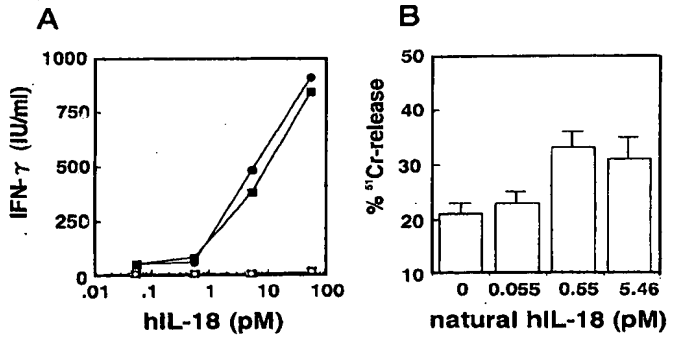


FIG. 3. **Biological activities of natural hIL-18 on human PBMC.** *Panel A*, induction of IFN-γ production by PBMC in response to hIL-18. Human PBMC (5×10^6 cells/ml) from healthy donors were incubated with various concentrations of natural or recombinant hIL-18 in the presence or absence of 0.1 μg/ml anti-CD3 mAb for 24 h. The amounts of IFN-γ produced in the culture supernatants were assessed by human IFN-γ ELISA. *Closed circles* and *closed squares* indicate the response to natural and recombinant hIL-18 in the presence of anti-CD3 mAb; *open circles* and *open squares* indicate the response to natural and recombinant hIL-18 without anti-CD3 mAb, respectively. *Panel B*, enhancement of NK cell cytotoxicity in response to natural hIL-18. Human PBMC (3×10^6 cells/ml) were incubated with various concentrations of natural hIL-18 for 48 h and then assayed for lytic activities against K562 target cells. The result shown was obtained at an effector:target ratio of 6:1. Each value is presented as a mean ± S.D. ($n = 3$).

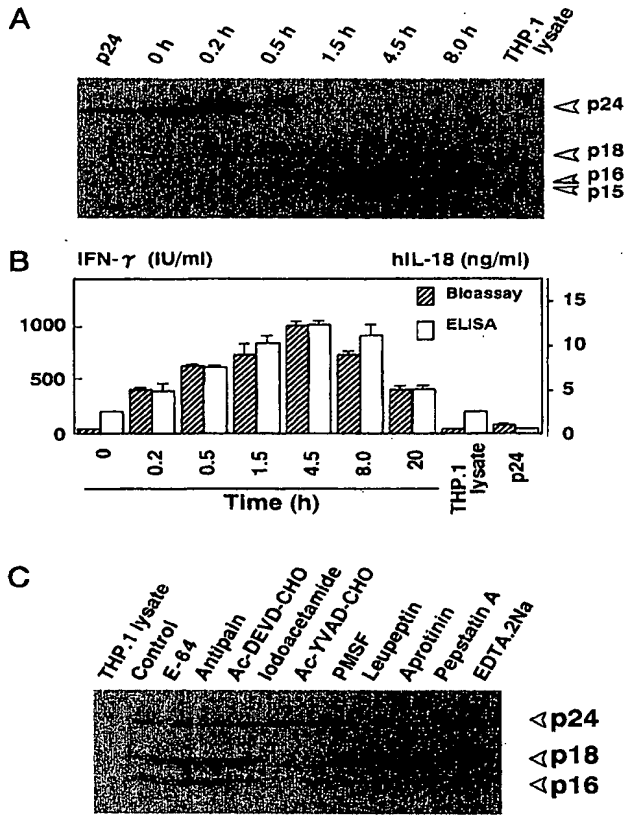


FIG. 4. ***In vitro* prohIL-18 cleavage assays using THP.1 cell lysate.** *Panel A*, a preparation containing about 200 ng of prohIL-18 was incubated with THP.1 cell lysate (30 μl) at 37 °C for the indicated periods in the presence of 20 mM Hepes, 10% glycerol, and 2 mM DTT, at pH 7.4 in a total volume of 50 μl. Cleaved products were analyzed by a standard immunoblotting method with anti-hIL-18 mAb (25-2G). Blots were developed using enhanced chemiluminescence (ECL) detection reagents (Amersham international, Buckinghamshire, U. K.). *Panel B*, hIL-18 activities of the reaction mixtures were assessed by IFN-γ-inducing assay on KG-1 cells (*hatched columns*) and hIL-18 ELISA (*open columns*). Each value is presented as a mean ± S.D. ($n = 3$). *Panel C*, various protease inhibitors were added to the prohIL-18 cleavage reactions and incubated for 2.5 h under the same conditions as described in *panel A*. Blots were developed using ECL detection reagents. Final concentrations of inhibitors used were: E64, 30 μM; antipain, 75 μM; Ac-DEVD-CHO, 10 μM; iodoacetamide, 650 μM; Ac-YVAD-CHO, 5 μM; phenylmethylsulfonyl fluoride (*PMSF*), 1 mM; leupeptin, 1 μM; aprotinin, 0.3 μM; pepstatin A, 1 μM; EDTA·2Na, 1.5 mM; control, without inhibitor.

(p16), and 15 kDa (p15), respectively (Fig. 4*A*). p18 can be detected within the first 30 min and subsequently, p16 and p15 appeared sequentially. Prolonged incubation resulted in a disappearance of the prohIL-18 band and in a decreased intensity of the p18 band. In contrast, an increase in the intensity of the p16 and p15 bands was observed. No significant proteolysis of these p16 and p15 bands was observed upon prolonged incubation, suggesting that there are at least three cleavage sites in prohIL-18 acting as substrates for the processing protease(s). In the same experiment, hIL-18 activities were measured by a hIL-18-specific ELISA (15) and by IFN-γ-inducing assay on KG-1 cells (Fig. 4*B*). KG-1 cells produce IFN-γ in response to hIL-18, but not to IL-12.[3] hIL-18 activity correlated with the appearance of the p18 molecule. p24, p16, and p15 were hardly detectable by the ELISA and show little or no IFN-γ-inducing activity on KG-1 cells (Fig. 4*B*). Next, to determine whether proteolytic cleavage is caused by ICE family proteases or by granzyme B, various protease inhibitors were employed. As expected, proteolysis showed features characteristic of caspases. The generation of the processed products was only completely inhibited by a thiol-alkylation reagent, iodoacetamide. Ac-YVAD-CHO inhibited the generation of p18 selectively and had no effect on that of p16. In contrast to this, Ac-DEVD-CHO inhibited the generation of p16 and had no effect on that of p18 (Fig. 4*C*). These results suggest that there are at least two IL-18-processing enzymes in THP.1 cell lysate, one is an ICE-like protease generating p18, the mature form of hIL-18, and the other is a CPP32-like protease generating p16 and/or p15, inactive degraded products of hIL-18.

Separation of the Converting and Degrading Enzymes—To confirm the above findings further, we tried to separate the two hIL-18-processing activities, hIL-18-converting activity (ICA) and hIL-18-degrading activity (IDA), from the cytosolic extract of THP.1 cells. Purification methods were partially based on those reportedly used for the separation of ICE (12, 14). Two activities were distinctly separated by DEAE-5PW column chromatography (Fig. 5, *insets*). ICA eluted sharply at a low NaCl concentration, whereas IDA eluted at a relatively high NaCl concentration as a broad peak. We also examined the ICE-like and CPP32-like activities (14, 18) in the DEAE elutes using the fluorogenic substrates Ac-YVAD-MAC and Ac-DEVD-MAC, respectively (Fig. 5). The ranges of activities of these two fluorogenic assays coincided with those of ICA and IDA as assessed by *in vitro* prohIL-18 cleavage assay, although high concentrations of IDA also exhibited Ac-YVAD-MAC cleavage activity. This cleavage of Ac-YVAD-MAC by IDA was confirmed using further purified preparations containing high concentrations of IDA (data not shown). Therefore, for further purification of ICA and IDA, we employed both ICE-like and CPP32-like cleavage assays on fluorogenic substrates and the *in vitro* prohIL-18 cleavage assay.

hIL-18-converting Enzyme Is ICE—hIL-18-CE was partially purified by S-Sepharose cation-exchange column chromatography, Mono S column chromatography followed by Superdex 200 column chromatography (data not shown). The fractions containing hIL-18-CE from Superdex 200 chromatography were

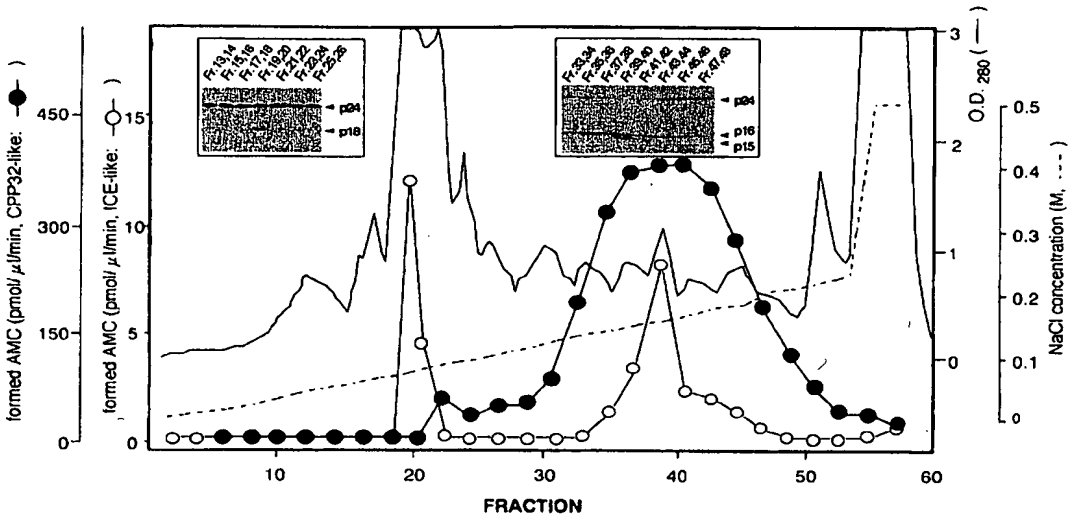


FIG. 5. **Separation of two types of hIL-18-processing activities which coincided with ICE-like and CPP32-like activities.** The precipitate from 80% saturated ammonium sulfate fractionation was dialyzed against HGCD buffer and eluted in a 550-ml DEAE-5PW column as described under "Experimental Procedures." hIL-18-processing activities in the fractions were detected by prohIL-18 cleavage assay (*insets*). ICE-like and CPP32-like activities in the fractions were measured as outlined under "Experimental Procedures." ICA (*left inset*, fractions 19 and 20) coincided with ICE-like activities (*open circles*), and IDA (*right inset*; fractions 33–48) coincided with CPP32-like activities (*closed circles*). A_{280} absorption and NaCl concentrations are shown as the *solid line* and the *dashed line*, respectively.

subjected to NH_2-terminal amino acid sequence analysis. From this analysis, both the large and small subunits of ICE (p20 and p10) were detected at about an equal molar ratio (Table II). The existence of these two subunits in the preparation was also confirmed by immunoblotting using anti-p10 and anti-p20 antibodies, respectively (data not shown). This suggested that hIL-18-converting enzyme may be ICE itself. To confirm this possibility, *in vitro* cleavage assays of precursor human IL-1β by partially purified hIL-18-CE were performed. hIL-18-CE could cleave precursor human IL-1β to generate the 17.5-kDa mature IL-1β as well as the mature hIL-18 (Fig. 6*A*). These cleavage activities were inhibited by Ac-YVAD-CHO and iodoacetamide. The bioactivity of the mature proteins was confirmed by IFN-γ-inducing assay for hIL-18 and D10.G4.1 proliferation assay (19) for IL-1β (data not shown). Furthermore, recombinant hICE cleaved prohIL-18 to generate the mature hIL-18 (Fig. 6*B*). These results support the conclusion that hIL-18-converting enzyme is ICE.

hIL-18-degrading Enzyme Is CPP32—hIL-18-degrading enzyme (hIL-18-DE) was partially purified by S-Sepharose cation-exchange column chromatography, Mono P chromatofocusing, Superdex 200, followed by hydroxyapatite column chromatographies (data not shown). Some members of the caspases, particularly those of the CPP32 subfamily, are reported to cleave poly(ADP-ribose)polymerase efficiently (18, 20, 21). Therefore, we tested this cleavage ability using partially purified hIL-18-DE. THP.1 cell-derived hIL-18-DE cleaved endogenous poly(ADP-ribose)polymerase to generate an NH_2-terminal 30-kDa fragment as well as degraded prohIL-18 (Fig. 7*A*). The preparation containing partially purified hIL-18-DE was also subjected to NH_2-terminal amino acid sequence analysis. From this analysis, both the large and small subunits of CPP32 (p17 and p12) and the small subunit of CMH-1 (p12) were detected (Table II). The quantities of these two enzymes were estimated to be comparable. Immunoblotting analyses also showed that both CPP32 and CMH-1 do indeed exist in the preparation containing hIL-18-DE (data not shown). These results suggested that the candidate for the hIL-18-degrading enzyme was either CPP32 or CMH-1. Cleavage assays using recombinant enzymes showed that effective degradation of prohIL-18 is caused by CPP32 but not by CMH-1 (Fig. 7*B*). CPP32 cleaved prohIL-18 >125-fold and >25-fold more efficiently than CMH-1 at pH 6.5 and pH 7.2, respectively (data not shown). From these results, we conclude that hIL-18-DE found in THP.1 cell lysate is CPP32.

DISCUSSION

Natural hIL-18 and its processing enzymes were identified in the cytosolic extract of monocytic THP.1 cells. It has been reported that murine IL-18 is produced by activated Kupffer cells (2) or intestinal epithelial cells (22). Fully activated Kupffer cells are considered to be able to secrete murine IL-18 efficiently with the aid of ICE (7). At the mRNA level, IL-18 is constitutively expressed in Kupffer cells, but the levels of IL-18 mRNA failed to elevate after injection of *P. acnes* intraperitoneally.[4] In our study, to identify hIL-18-producing cell lines, constitutive expression of hIL-18 mRNA in 42 hematopoietic cell lines was examined. High levels of mRNA expression were detected predominantly in myelomonocytic and non-L, non-M cell lines. Some B cell and non-T, non-B lines showed low expression of IL-18 mRNA, whereas T cell lines did not show any expression at all. These results strongly support the notion that major IL-18-producing cells are macrophage-like cells.

During purification of natural hIL-18, endogenous precursor protein or probable degraded products were detected by immunoblotting analysis, from which the mature hIL-18 was isolated selectively by specific affinity chromatography (Fig. 1*C*). In respect of the enhancement of NK cell cytotoxicity and IFN-γ production by human PBMC, purified natural hIL-18 possessed the same biological functions as those of the recombinant protein that had an NH_2-terminal sequence tentatively assigned based on homology with the murine natural IL-18 (Fig. 2). In fact, the maturation site of natural hIL-18 was in accordance with that of the murine homolog. hIL-18 has no putative *N*-linked glycosylation site, and the theoretical molec-

[4] Tsutsui, H., Matsui, K., Kawada, N., Hyodo, Y., Hayashi, N., Okamura, H., Higashino, K., and Nakanishi, K. (1997) *J. Immunol.*, in press

TABLE II

NH_2-terminal amino acid sequences of hIL-18-processing enzymes detected in partially purified preparations

The preparations containing partially purified hIL-18-CE and hIL-18-DE were subjected to SDS-PAGE analysis followed by direct NH_2-terminal sequencing as described under "Experimental Procedures." The (–) denotes a residue that could not be assigned based on peptide sequence analysis.

Polypeptide	Amino acid sequence	Assigned protein
hIL-18-CE		
22 kDa	D/N[a] P A M P T – – G – E G N V K L – – L	hICE-p20
10 kDa	A I K K A H I E K D F I A F	hICE-p10
hIL-18-DE		
17 kDa	S G I K L D N – Y K M – Y P E M – – I – I	hCPP32-p17
12 kDa	S G V D D D M A – H	hCPP32-p12
11 kDa	A – P R Y K I P V E A	hCMH-1-p12

[a] Two amino acids (Asp and Asn) were detected in the first cycle of the Edman sequencing.

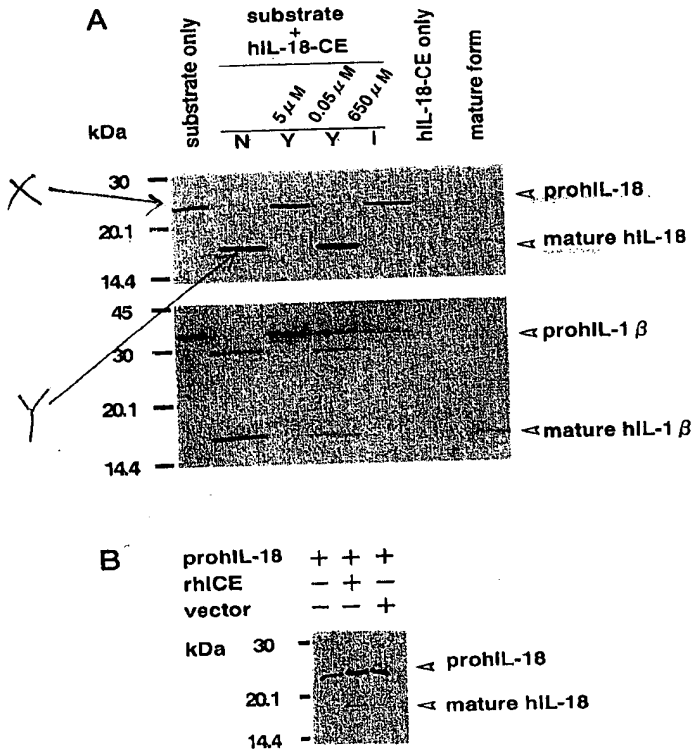


FIG. 6. ***In vitro* cleavage assays for the identification of hIL-18-CE.** *Panel A*, a preparation of prohIL-18 or prohIL-1β (about 100 ng each) were incubated at 37 °C for 3 h with partially purified hIL-18-CE (30 μl) in the presence of 20 mM Hepes, 10% glycerol, 2 mM DTT, at pH 7.4 in a total reaction volume of 50 μl. *I*, *Y*, and *N* denote iodoacetamide, Ac-YVAD-CHO, and without inhibitor, respectively. Cleaved products were analyzed by immunoblotting with anti-hIL-18 mAb (25-2G) or anti-hIL-1β pAb and visualized by enhanced chemiluminescence detection reagents. *Panel B*, a preparation containing about 100 ng of prohIL-18 was incubated with partially purified recombinant hICE (30 μl) with the same conditions as in *panel A*. *Vector* denotes the enzymatic preparation from COS-1 cells transfected with control vector (pCDM8). Cleaved products were visualized by immunoblotting as in Fig. 4*A*.

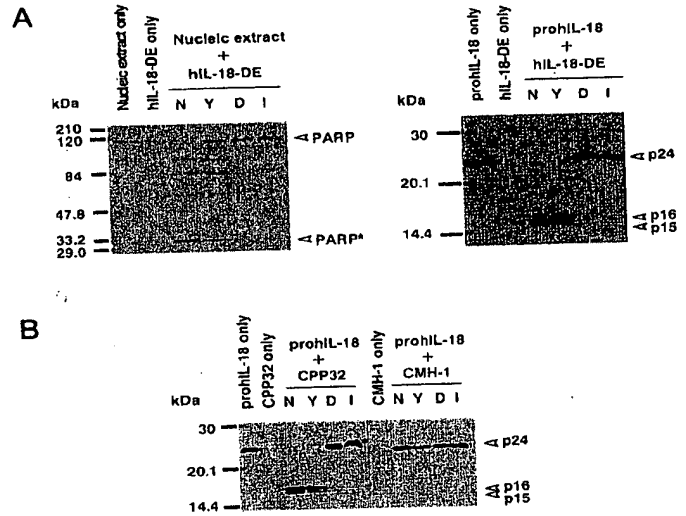


FIG. 7. ***In vitro* cleavage assays for the identification of hIL-18-DE.** *Panel A*, nucleic extract containing poly(ADP-ribose)polymerase was prepared from THP.1 cells by the method of Sadowski and Gilman (36). Nucleic extract (2 μl) from THP.1 cells was incubated at 37 °C for 10 min with the partially purified hIL-18-DE (5 μl) under the same conditions. *PARP** denotes the degraded form of poly(ADP-ribose)polymerase (*left*). A preparation containing about 100 ng of prohIL-18 was incubated at 37 °C for 2 h with partially purified hIL-18-DE (10 μl) with 20 mM Hepes, 10% glycerol, 2 mM DTT, at pH 6.5 in a total of 20 μl of reaction volume (*right*). *D*, *Y*, *I*, and *N* denote Ac-DEVD-CHO (10 μM), Ac-YVAD-CHO (10 μM), iodoacetamide (650 μM), and without inhibitor, respectively. Cleaved products were analyzed by immunoblotting with anti-hIL-18 mAb (25-2G) or anti-human poly(ADP-ribose)polymerase pAb, and enhanced chemiluminescence detection reagents. *Panel B*, a preparation containing about 100 ng of prohIL-18 was incubated at 37 °C for 2 h with 50 nM purified recombinant human CPP32 or CMH-1 in the presence of 20 mM Hepes, 10% glycerol, and 2 mM DTT at pH 6.5 in a total reaction volume of 10 μl. Cleaved products were visualized by immunoblotting as in Fig. 4*A*.

ular mass deduced from the genetic information is 18,217 Da. The molecular mass of natural hIL-18 determined by SDS-PAGE analysis is 18.2 kDa (Fig. 1*C*). These results suggest that THP.1-derived natural hIL-18 is a monomeric 18-kDa protein without a sugar chain or post-transcriptional modification.

Natural prohIL-18 is detected as a 24-kDa band by SDS-PAGE analysis under reducing conditions, although the theoretical molecular mass of prohIL-18 is 22,326 Da. Moreover, it seems likely that the NH_2 terminus of natural prohIL-18 is blocked by unknown modification from the finding that the NH_2-terminal amino acid could not be analyzed by the direct sequencing method (data not shown). These results suggest that prohIL-18 may be modified to facilitate its constitutive presence in the cell. This further implies that specific secretion mechanisms may be active in macrophage-like cells to transport mature IL-18 into the extracellular space similarly to IL-1β. However, preliminary studies on hIL-18 mRNA-expressing cell lines failed to detect extracellular hIL-18 even under the same conditions that IL-1β secretion is observed (data not shown). This might be due only to the lack of accumulation of the precursor protein in the cytoplasm. Another possibility is that the regulatory mechanisms for secretion may be different between hIL-18 and IL-1β. The latter assumption is supported by the hypothesis on the multistage regulation of IL-1β secretion proposed by Siders and Mizel (23).

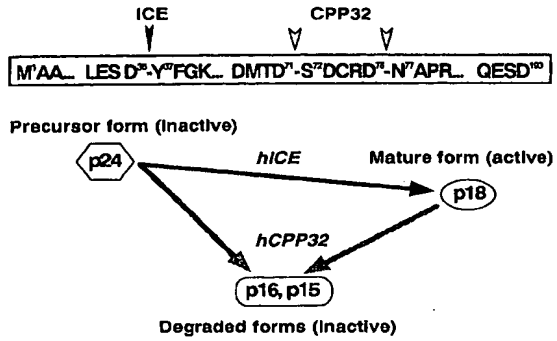


FIG. 8. **Schematic illustration of cleavage sites in hIL-18.** hIL-18 is synthesized as a biologically inactive 24-kDa precursor polypeptide (p24). LESD^{36}-Y^{37} is the maturation site for p24 cleaved by ICE to generate the active p18 molecule, and D*X*XD-*X*s (DMTD^{71}-S^{72} and DCRD^{76}-A^{77}) are the cleavage sites for CPP32 resulting in the generation of biologically inactive p16 and p15 products. All three cleavage sites for processing enzymes in hIL-18 were determined by NH_2-terminal amino acid sequence analysis of the cleaved products.

With respect to the structural similarity between IL-18 and IL-1β, the IL-1 signature-like sequence (3) and structural similarities including the lack of signal-peptide sequences have been reported (4). Moreover, involvement of ICE/caspase-1 in the maturation of endogenous hIL-18 has also been confirmed in this report. However, the biological functions of IL-18 show clear differences from those of IL-1β in terms of the augmentation of NK cell cytotoxicity and IFN-γ and granulocyte-macrophage colony-stimulating factor production by T cells and the induction of T cell proliferation (3, 24). Furthermore, although IL-1β is known to act as an accessory molecule in the activation of Th2 cells (25), IL-18 reportedly acts on Th1 cells but not on Th2 cells to augment IFN-γ production and induces proliferation and IL-2 receptor α-chain expression of Th1 cells (26). Thus, IL-18 and IL-1β exhibit different biological functions transmitted through their specific receptors.[2]

THP.1 is well known as an ICE- and CPP32-producing cell line (14, 18). Therefore, it is not surprising that these two enzymes were identified in THP.1 cell-free extracts. Of great interest is that a common substrate of ICE and CPP32 was found concurrently. Fig. 8 shows the schematic illustration of experimentally determined cleavage sites for hIL-18 by ICE and CPP32. As described above, cleavage by ICE occurs at Asp^{36}-Tyr^{37}. This cleavage site is reportedly recognized also by ICH-2/caspase-4 and ICE-rel III/caspase-5 but much less efficiently than ICE/caspase-1 (8). The recognition site for ICE cleavage and maturation of hIL-18 differs markedly from that of IL-1β, LESD-Y for hIL-18 and YVHD-N for IL-1β, respectively. Many studies have been performed to date based on this tetrapeptide sequence motif to design ICE inhibitors for therapeutic usage (14, 27–32). The LESD motif also might become a useful candidate for the generation of an effective ICE inhibitor.

In the case of CPP32, cleavage sites for hIL-18 degradation are Asp^{71}-Ser^{72} and Asp^{76}-Asn^{77}, resulting in the generation of inactive p16 and p15, respectively. This indicates that the NH_2-terminal portion of the mature hIL-18 (Tyr^{37}-Asp^{71}) is indispensable for the expression of its bioactivity. The D*X*XD-*X* motif is known to be a specific target sequence for cleavage by the CPP32 subfamily to which CPP32/caspase-3 (18), CMH-1/Mch3/caspase-7 (21, 33), and Mch2/caspase-6 (20) belong. Moreover, *in vitro* cleavage assays using mature hIL-18 revealed that CPP32 cleaved not only prohIL-18 but also mature hIL-18 as a substrate to generate the same biologically inactive products, p16 and p15 polypeptides (data not shown). These results suggest that CPP32 may act as a potential down-regulator of IL-18.

We observed these enzymatical cleavages in *in vitro* cell-free systems. Further experiments are therefore necessary to address physiological processing or secretion mechanisms for IL-18. However, under physiological conditions, it is likely that proIL-18 and proIL-1β may compete with each other as substrates for endogenous active ICE. It is reported that proIL-1β seems to act as an endogenous competitive inhibitor against cleavage of other "death substrates" by ICE in Fas-mediated apoptosis (34). proIL-18 may compete or cooperate with proIL-1β in such an apoptotic event. In addition, Fas-mediated apoptosis induces sequential activation of ICE-like and CPP32-like enzymes in murine thymocytes (35). It seems possible that endogenous prohIL-18 and/or mature hIL-18 also might be linked to the sequential activation of these caspases during apoptotic events. The fact that two caspases (ICE and CPP32) which might act sequentially could operate the "activation/inactivation switch," for IL-18 is a very attractive model for further study on physiological roles of IL-18.

In conclusion, the analyses on natural hIL-18 purified from monocytic THP.1 cells have revealed the involvement of endogenous ICE/caspase-1 in its maturation. Moreover, endogenous CPP32/caspase-3 was found to degrade hIL-18 to biologically inactive forms effectively. Although further experiments are necessary to demonstrate the secretion mechanism or physiological regulation of IL-18, these findings will help to shed some light on the potential roles of IL-18 in immune responses.

Acknowledgement—We thank Dr. Mark Micallef for a helpful review of the manuscript and encouragement throughout this study. We also thank Mutsuko Taniguchi for anti-hIL-18 mAbs and anti-hIL-18-Sepharose; and Madoka Taniai, Shimpei Ushio, Dr. Kanso Iwaki, and Dr. Osamu Ando for helpful advice.

REFERENCES

1. Okamura, H., Nagata, K., Komatsu, T., Tanimoto, T., Nukada, Y., Tanabe, F., Akita, K., Torigoe, K., Okura, T., Fukuda, S., and Kurimoto, M. (1995) *Infect. Immun.* **63**, 3966–3972
2. Okamura, H., Tsutsui, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K., Okura, T., Nukada, Y., Hattori, K., Akita, K., Namba, M., Tanabe, F., Konishi, K., Fukuda, S., and Kurimoto, M. (1995) *Nature* **378**, 88–91
3. Ushio, S., Namba, M., Okura, T., Hattori, K., Nukada, Y., Akita, K., Tanabe, F., Konishi, K., Micallef, M., Fujii, M., Torigoe, K., Tanimoto, T., Fukuda, S., Ikeda, M., Okamura, H., and Kurimoto, M. (1996) *J. Immunol.* **156**, 4274–4279
4. Bazan, J. F., Timans, J. C., and Kastelein, R. A. (1996) *Nature* **379**, 591
5. Murzin, A. G., Lesk, A. M., and Chothia, C. (1992) *J. Mol. Biol.* **223**, 531–543
6. Dinarello, C. A. (1996) *Blood* **87**, 2095–2147
7. Gu, Y., Kuida, K., Tsutsui, H., Ku, G., Hsiao, K., Fleming, M. A., Hayashi, N., Higashino, K., Okamura, H., Nakanishi, K., Kurimoto, M., Tanimoto, T., Flavell, R. A., Sato, V., Harding, M. W., Livingston, D. J., and Su, M. S.-S. (1997) *Science* **275**, 206–209
8. Ghayur, T., Banerjee, S., Hugunin, M., Butler, D., Herzog, L., Carter, A., Quintal, L., Sekut, L., Talanian, R., Paskind, M., Wong, W., Kamen, R., Tracey, D., and Allen, H. (1997) *Nature* **386**, 619–623
9. Ando, S., Ohta, T., Tanimoto, T., Sano, O., Yamauchi, H., Ando, O., Torigoe, K., and Kurimoto, M. (1988) *Jpn. J. Cancer Res.* **79**, 757–765
10. Fukuda, S., Ando, S., Sanou, O., Taniai, M., Fujii, M., Masaki, N., Nakamura, K., Ando, O., Torigoe, K., Sugimoto, T., and Kurimoto, M. (1988) *Lymphokine Res.* **7**, 175–185
11. Kostura, M. J., Tocci, M. J., Limjuco, G., Chin, J., Cameron, P., Hillman, A. G., Chartrain, N. A., and Schmidt, J. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 5227–5231
12. Miller, D. K., Ayala, J. M., Egger, L. A., Raju, S. M., Yamin, T.-T., Ding, G. J.-F., Gaffney, E. P., Howard, A. D., Palyha, O. C., Rolando, A. M., Salley, J. P., Thornberry, N. A., Weidner, J. R., Williams, J. H., Chapman, K. T., Jackson, J., Kostura, M. J., Limjuco, G., Molineaux, S. M., Mumford, R. A., and Calaycay, J. R. (1993) *J. Biol. Chem.* **268**, 18062–18069
13. Karasuyama, H., Kudo, A., and Melchers, F. (1990) *J. Exp. Med.* **172**, 969–972
14. Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., Aunins, J., Elliston, K. O., Ayala, J. M., Casano, F. J., Chin, J., Ding, G. J.-F., Egger, L. A., Gaffney, E. P., Limjuco, G., Palyha, O. C., Raju, S. M., Rolando, A. M., Salley, J. P., Yamin, T.-T., Lee, T. D., Shively, J. E., MacCross, M., Mumford, R. A., Schmidt, J. A., and Tocci, M. J. (1992) *Nature* **356**, 768–774
15. Taniguchi, M., Nagaoka, K., Kunikata, T., Kayano, T., Yamauchi, H., Nakamura, S., Ikeda, M., and Kurimoto, M. (1997) *J. Immunol. Methods*, in press
16. Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvensen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996) *Cell* **87**, 171

17. Darmon, A. J., Nicholson, D. W., and Bleackley, R. C. (1995) *Nature* **377**, 446–448
18. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T.-T., Yu, V. L., and Miller, D. K. (1995) *Nature* **376**, 37–43
19. Kurt-Jones, E. A., Beller, D. I., Mizel, S. B., and Unanue, E. R. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 1204–1208
20. Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1995) *Cancer Res.* **55**, 2737–2742
21. Lippke, J. A., Gu, Y., Sarnecki, C., Caron, P. R., and Su, M. S.-S. (1996) *J. Biol. Chem.* **271**, 1825–1828
22. Takeuchi, M., Nishizaki, Y., Sano, O., Ohta, T., Ikeda, M., and Kurimoto, M. (1997) *Cell Tissue Res.*, **289**, 499–503
23. Siders, W. M., and Mizel, S. B. (1995) *J. Biol. Chem.* **270**, 16258–16264
24. Micallef, M. J., Ohtsuki, T., Kohno, K., Tanabe, F., Ushio, S., Namba, M., Tanimoto, T., Torigoe, K., Fujii, M., Ikeda, M., Fukuda, S., and Kurimoto, M. (1996) *Eur. J. Immunol.* **26**, 1647–1651
25. Lichtman, A. H., Chin, J., Schmidt, J. A., and Abbas, A. K. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 9699–9703
26. Kohno, K., Kataoka, J., Ohtsuki, T., Suemoto, Y., Okamoto, I., Usui, M., Ikeda, M., and Kurimoto, M. (1997) *J. Immunol.* **158**, 1541–1550
27. Thornberry, N. A., and Molineaux, S. M. (1995) *Protein Sci.* **4**, 3–12
28. Nicholson, D. W. (1996) *Nature Biotech.* **14**, 297–301
29. Thornberry, N. A., Peterson, E. P., Zhao, J. J., Howard, A. D., Griffin, P. R., and Chapman, K. T. (1994) *Biochemistry* **33**, 3934–3940
30. Walker, N. P. C., Talanian, R. V., Brady, K. D., Dang, L. C., Bump, N. J., Ferenz, C. R., Franklin, S., Ghayur, T., Hackett, M. C., Hammill, L. D., Herzog, L., Hugunin, M., Houy, W., Mankovich, J. A., McGuiness, L., Orlewicz, E., Paskind, M., Pratt, C. A., Reis, P., Summani, A., Terranova, M., Welch, J. P., Xiong, L., Möller, A., Tracey, D. E., Kamen, R., and Wong, W. W. (1994) *Cell* **78**, 343–352
31. Wilson, K. P., Black, J.-A. F., Thomson, J. A., Kim, E. E., Griffith, J. P., Navia, M. A., Murcko, M. A., Chambers, S. P., Aldape, R. A., Raybuck, S. A., and Livingston, D. J. (1994) *Nature* **370**, 270–275
32. Thornberry, N. A., Miller, D. K., and Nicholson, D. W. (1995) *Perspectives Drug Discovery Design* **2**, 389–399
33. Fernandes-Alnemri, T., Takahashi, A., Armstrong, R., Krebs, J., Fritz, L., Tomaselli, K. J., Wang, L., Yu, Z., Croce, C. M., Salvensen, G., Earnshaw, W. C., Litwack, G., and Alnemri, E. S. (1995) *Cancer Res.* **55**, 6045–6052
34. Tastuta, T., Cheng, J., and Mountz, J. D. (1996) *J. Immunol.* **157**, 3949–3957
35. Enari, M., Talanian, R. V., Wong, W. W., and Nagata, S. (1996) *Nature* **380**, 723–726
36. Sadowski, H. B., and Gilman, M. Z. (1993) *Nature* **362**, 79–83